

cient strain, AB309-105, is used in comparison to wild-type RNaseIII-containing bacteria, BL21(DE3).

Transformation of AB309-105 and BL21(DE3)

[0455] Three hundred ng of the plasmid are added to and gently mixed in a 50 μ A aliquot of ice-chilled chemically competent *E. coli* strain AB309-105 or BL21(DE3). The cells are incubated on ice for 20 minutes before subjecting them to a heat shock treatment of 37° C. for 5 minutes, after which the cells are placed back on ice for a further 5 minutes. Four hundred and fifty μ l of room temperature SOC medium is added to the cells and the suspension incubated on a shaker (250 rpm) at 37° C. for 1 hour. One hundred μ l of the bacterial cell suspension is transferred to a 500 ml conical flask containing 150 ml of liquid Luria-Bertani (LB) broth supplemented with 100 μ g/ml carbenicillin antibiotic. The culture is incubated on an Innova 4430 shaker (250 rpm) at 37° C. overnight (16 to 18 hours).

Chemical Induction of Double-Stranded RNA Expression in AB309-105 and BL21(DE3)

[0456] Expression of double-stranded RNA from the recombinant vector, pGBNJ003, in the bacterial strain AB309-105 or BL21(DE3) is made possible since all the genetic components for controlled expression are present. In the presence of the chemical inducer isopropylthiogalactoside, or IPTG, the T7 polymerase will drive the transcription of the target sequence in both antisense and sense directions since these are flanked by oppositely oriented T7 promoters.

[0457] The optical density at 600 nm of the overnight bacterial culture is measured using an appropriate spectrophotometer and adjusted to a value of 1 by the addition of fresh LB broth. Fifty ml of this culture is transferred to a 50 ml Falcon tube and the culture then centrifuged at 3000 g at 15° C. for 10 minutes. The supernatant is removed and the bacterial pellet resuspended in 50 ml of fresh S complete medium (SNC medium plus 5 μ g/ml cholesterol) supplemented with 100 μ g/ml carbenicillin and 1 mM IPTG. The bacteria are induced for 2 to 4 hours at room temperature.

Heat Treatment of Bacteria

[0458] Bacteria are killed by heat treatment in order to minimise the risk of contamination of the artificial diet in the test plates. However, heat treatment of bacteria expressing double-stranded RNA is not a prerequisite for inducing toxicity towards the insects due to RNA interference. The induced bacterial culture is centrifuged at 3000 g at room temperature for 10 minutes, the supernatant discarded and the pellet subjected to 80° C. for 20 minutes in a water bath. After heat treatment, the bacterial pellet is resuspended in 1.5 ml MilliQ water and the suspension transferred to a microfuge tube. Several tubes are prepared and used in the bioassays for each refreshment. The tubes are stored at -20° C. until further use.

F. Laboratory Trials to Test *Escherichia coli* Expressing dsRNA Targets Against *Acheta domesticus*

Plant-Based Bioassays

[0459] Whole plants are sprayed with suspensions of chemically induced bacteria expressing dsRNA prior to feeding the plants to HC. The are grown from in a plant growth

room chamber. The plants are caged by placing a 500 ml plastic bottle upside down over the plant with the neck of the bottle firmly placed in the soil in a pot and the base cut open and covered with a fine nylon mesh to permit aeration, reduce condensation inside and prevent insect escape. HC are placed on each treated plant in the cage. Plants are treated with a suspension of *E. coli* AB309-105 harbouring the pGBNJ001 plasmids or pGN29 plasmid. Different quantities of bacteria are applied to the plants: for instance 66, 22, and 7 units, where one unit is defined as 10⁹ bacterial cells in 1 ml of a bacterial suspension at optical density value of 1 at 600 nm wavelength. In each case, a total volume of between 1 and 10 ml is sprayed on the plant with the aid of a vaporizer. One plant is used per treatment in this trial. The number of survivors are counted and the weight of each survivor recorded.

[0460] Spraying plants with a suspension of *E. coli* bacterial strain AB309-105 expressing target dsRNA from pGBNJ003 led to a dramatic increase in insect mortality when compared to pGN29 control. These experiments show that double-stranded RNA corresponding to an insect gene target sequence produced in either wild-type or RNaseIII-deficient bacterial expression systems is toxic towards the insect in terms of substantial increases in insect mortality and growth/development delay for larval survivors. It is also clear from these experiments that an exemplification is provided for the effective protection of plants/crops from insect damage by the use of a spray of a formulation consisting of bacteria expressing double-stranded RNA corresponding to an insect gene target.

Example 13

Pyricularia grisea (Rice Blast)

A. Cloning *P. grisea* Partial Sequences

[0461] High quality, intact RNA is isolated from different growth stages of *P. grisea* using TRIzol Reagent (Cat. Nr. 15596-026/15596-018, Invitrogen, Rockville, Md., USA) following the manufacturer's instructions. Genomic DNA present in the RNA preparation is removed by DNase treatment following the manufacturer's instructions (Cat. Nr. 1700, Promega). cDNA is generated using a commercially available kit (SuperScript™ III Reverse Transcriptase, Cat. Nr. 18080044, Invitrogen, Rockville, Md., USA) following the manufacturer's instructions.

[0462] To isolate cDNA sequences comprising a portion of a target gene, PCR is performed with degenerate primers using Amplitaq Gold (Cat. Nr. N8080240, Applied Biosystems) following the manufacturer's instructions. The resultant PCR products are fractionated and sequenced.

B. dsRNA Production of *P. grisea* Genes

[0463] dsRNA is synthesized in milligram amounts using a commercially available kit, such as T7 Ribomax™ Express RNAi System (Cat. Nr. P1700, Promega), following the manufacturer's instructions. The resulting PCR products are analyzed on an agarose gel and purified by a PCR purification kit (e.g. Qiaquick PCR Purification Kit, Cat. Nr. 28106, Qiagen) and NaClO₄ precipitation. The product T7 forward and reverse templates are mixed and the resulting RNA strands are annealed, then DNase and RNase treated, and purified by sodium acetate, following the manufacturer's instructions.